PECVD coatings for functionalization of Point-Of-Care biosensor surfaces

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Abstract

In early stage disease diagnosis, an accurate and reliable measurement of low concentrations of specific biomarkers is a key need. The detection technique requires the reaction of an antibody, which is generally covalently bound to the biosensor platform, with its antigen. The application of Zeonor®, a cyclo olefin copolymer (COP) with very low autofluorescence, good optical properties and high precision molding characteristics, as a biosensor platform has been demonstrated recently. Highly reproducible, industrial scale surface chemical modification of the COP plastic for covalent attachment of the biomolecules for specific recognition of the target, together with low non specific binding of other proteins that may be present in the sample is a key challenge. In this work, the applicability of plasma enhanced chemical vapor deposition (PECVD) process has been demonstrated by depositing varying surface functionalities including amines, carboxylic, mercapto, epoxy and polyethylene glycol functionalities. The plasma functionalized coatings thus created possess both reactive and repellent sites on the biosensor chip, allowing the chip to be configured either for fluorescence or light scattering–based detection or for label-free surface plasmon resonance detection

techniques. The versatility of the gas phase deposition process for building sequential chemistries on low cost and disposable plastic chips is presented in detail.

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1. Introduction

With the recent advances in the synthesis of new polymeric materials and increased interest in point-of-care applications, significant effort has been invested to develop cheap and disposable biosensor platforms, with excellent physical properties, good chemical resistance and ease of fabrication. The technological processes required for the manufacture of the disposable substrates, often with microfluidic features such as micro-channels, pillars and micro valves among others, have been successfully mastered to some extent [1]. In a typical format of bioassay device, a biorecognition element, such as antibody or oligonucleotide, must be immobilized on a substrate's surface [2]. The mode of the immobilization must allow for efficient binding to the desired analyte. This binding event is then detected and visualized by the detection technique directly or may require another tagged-element to recognize the analyte in an additional step. It is therefore understandable that for successful measurement, the biggest challenge is to obtain maximum possible signal when the analyte is present in the measured sample and minimum possible signal in the absence of the analyte, also called non-specific binding [3]. The ratio between the signal to background defines the sensitivity of the device. It is not surprising that the surface, onto which the biorecognition elements are immobilized, plays a key role in the performance of the diagnostic device. A carefully executed surface chemistry can be fine-tuned to maximize the signal and minimize the noise contribution, or ideally both. This article will therefore focus on strategies to functionalize plastic, disposable substrates, used in biomedical diagnostics devices.

Pristine polymeric materials, like cyclo olefin polymer (COP), from which the substrates are typically made of, are not very suitable for capturing antibodies or single strand DNA molecules in their native, active form. Functionalization of such surface is therefore

necessary. Oxygen plasma or UV/ozone treatment results in a formation of thin, oxidized and hydrophilic layer, which can be used to adsorb proteins and oligonucleotides [4-6]. However, this film contains many highly reactive and unstable oxidized species that are readily quenched by any impurities and gas molecules present in the air. Moreover, such a prepared surface offers only a low degree of flexibility of the functional groups, which may be critical for proper orientation of the captured biomolecules. A typical solution is to functionalize the plastic substrate with organic compounds that can cross-link, polymerize and form a film, from one to a few hundreds of nanometers in thickness [7-9]. This film should contain appropriate functional groups that can be either directly or with the aid of other small molecules used to capture the biorecognition elements in high density and retain their activity towards the analyte of interest. From a chemical perspective, the most useful functional groups on a substrate are the ones with good reactivity towards amines, carboxylic acids and thiols. These three functionalities are some of the most abundant and reactive in proteins and can be relatively easily incorporated into an oligonucleotide.

Deposition of inorganic pin hole free uniform coatings like silicon-dioxide, by plasma enhanced chemical vapor deposition (PECVD) has been demonstrated which has an extensive application in biomedical, food packing and scratch resistance properties [10-12]. PECVD of organic functional groups [13; 14] for biomolecule immobilization in biosensors and for anti-fouling characteristics is an area requiring intense research. Very recently, PECVD has also been used for producing hybrid organic-inorganic structures for gate oxides in bioanalytical field effect transistors [15]. The physical and chemical properties of the films deposited by PECVD are dependent on plasma process parameters. The effect of ion bombardment on the growing film and the plasma electron density are crucial and could lead to dissociation of functional groups in the plasma as well as on the growing film. In this article, synthetic approaches for modification of COP substrates with $–NH_2$ and –SH functional groups will be discussed. Perhaps more importantly, we will also provide a review on the analytical tools used to assess the quality of the prepared films. For a design of sensitive bioassay device, it is critical to understand interaction forces in real systems, and how these relate on the one hand to the surface composition and chemistry, and on the other hand to the specificity, sensitivity and resistance to non-specific binding.

2. Materials and methods

2.1 Plasma Enhanced Chemical Vapor Deposition

The vacuum chamber is an aluminum based container. The pressure in the chamber was measured using a Granville-Phillips gauge. An Edwards EH mechanical booster pump backed by an Edwards E1M40 rotary pump was used to pump down the chamber. The rotary pump was connected through a port at the bottom of the chamber.
The powered electrode, 24cm x 21cm plate with a 6cm diameter with a hole in the middle, was placed slightly below the top of the chamber and the chamber wall was grounded. The powered electrode is cooled with running water. Also a 24cm X 21cm X 1.2cm electrically isolated, water cooled hollow metallic setup placed 10 cm away from the powered electrode is used as the substrate holder. The powered electrode (PE) is separated from the ground chamber by ceramic spacers and a floating potential (FP) electrode is placed under the powered electrode. During the process, electric potential from the radio-frequency (RF) generator is applied to the powered electrode, which in turn excites the gases present in the chamber to a plasma state. The RF generator was connected to the chamber through an automated matching box. The mass flow controllers (MFC) were used to control the flow of gases. Before each MFC, a shut off valve was installed to avoid leakage of any gas that was not used during the process.

2.2 Materials

A type of cyclo olefin polymer sold under the trade name of Zeonor® was obtained from Amic AB (Uppsala, Sweden). 3-aminopropyltriethoxysilane (APTES), ethylenediamine (EDA), diethylene glycol dimethyl ether (DEGDME), 3-Mercaptopropyltrimethoxysilane (MPTMS), acetonitrile and triethylamine were purchased from Sigma Aldrich and used without further treatment. Lissamine rhodamine B sulfonyl chloride ($\lambda_{exc.}$ = 532-nm excitation and λ_{em} = 550-nm) was obtained from Invitrogen.

2.3 Coatings preparation

Zeonor® slides were cleaned with dry air and then loaded in the chamber. The chamber was pumped down to a base pressure of 20 mTorr. Prior to the deposition, plasma cleaning and activation was carried out using argon (50 sccm) + oxygen (50sccm) mix plasma (250 Watts RF power). After three minutes, the oxygen flow is closed and the RF power reduced to 14Watts. The deposition time was 4 minutes. APTES, EDA, DEGDME, MPTMS precursors were stored in a container (connected to the vacuum chamber). A needle valve was used to control the flow of precursor vapors. The vapor pressures of APTES and MPTMS being higher than that of EDA and DEGDME, the source and the pipeline containing APTES and MPTMS were heated to about 80 and 60 degrees respectively.

2.4 Characterization Techniques

This section aims to give the reader an overview of the techniques available for the characterization of films deposited by PECVD. The measurement principle and specific film property measured is discussed to aid in the selection of a technique suited to a particular application. Techniques are included which measure physical film properties, chemical properties (both elemental and chemical) and which probe for the availability of specific chemical groups at the film surface.

2.4.1 Ellipsometry

Ellipsometry [16] is a very effective and non-destructive method to characterize the physical properties, i.e. refractive index and thickness, of a thin film on a reflecting substrate. Ellipsometry works by detecting the change in polarization of light before going through and after reflecting off the substrate. The change in polarization for a specific film and substrate at a specific incident angle is represented by two ellipsometric angles, Ψ and Δ versus the range of wavelength of interest. These two ellipsometric angles, Ψ and Δ can be used to deduce the thickness of the film of known refractive index with the help of mathematical modeling and fitting. This technique provides a

simple and rapid measurement for film thickness. It is particularly useful for evaluation of coverage uniformity by taking measurements at multiple positions around a sample deposited by PECVD [17; 18]. The film thickness was measured by using J.A. Woollam Co., Inc. M-2000UI spectroscopic ellipsometer.

2.4.2 Fluorescence Scanning/Microscopy

Fluorescence scanning or microscopy can be used to highlight specific functional groups (e.g. amino) present on the film surface. The film is incubated with a fluorophore functionalized to form a chemical bond with a particular group expected to be present on the film. A fluorescence image of the film is then generated with either a GMS 418 scanner (Genetic Microsystems, Affymetrix) or microscope (Cell R Olympus system) to visually evaluate the spatial intensity.

2.4.3 Contact Angle

Film wettability can be analyzed by measuring the water contact angle of the film surface. As the addition of a film typically causes a significant change to the surface hydrophobicity, this simple measurement can give information about the film coverage. Film stability and ageing can also be evaluated by repeatedly measuring the contact angle over time.

2.4.4 Secondary Ion Mass Spectrometry (SIMS)

Secondary Ion Mass spectrometry (SIMS) is, in spite of its destructive nature, one of the most appealing characterization methods, thanks to its ability to provide specific chemical information about a film as a function of depth. This information is obtained by bombardment of the film with a perpendicular focused ion beam. This bombardment causes the release of secondary ions, which are analyzed for molecular fragmentation as in conventional mass spectrometry.

The secondary ion mass spectroscopic studies were carried out using a quadrupole apparatus MiniSIMS developed by Millbrook Instruments Ltd. It incorporates a raster scanned gallium liquid metal ion gun for the primary beam and low-energy optics for secondary ion extraction into a 300 Da quadrupole. Ga+ ions (6 keV) were focussed perpendicularly to the substrate. To mitigate charging of the electrically insulating Zeonor substrate an electron gun charge neutralisation was used for all the measurements. The operating pressure was 3.1×10^{-7} mbar and the chemical imaging was performed in a broad beam mode.

2.4.5 UV/Vis Colorimetry Using sSDTB

Colorimetry can be used in combination with chemical processing to determine the number of amine groups present at a film surface. The film is incubated in a solution of sulfosuccinimidyl- 4-[2-(4,4-dimethoxytrityl)]butyrate (sSDTB) then rinsed with water and treated with perchloric acid to allow the formation of 4,40-dimethoxytrityl cation from the substrates. Since the reaction between the amines and the sSDTB proceeds with 1:1 stoichiometric ratio, the concentration of the released cation measured by UV/vis spectrophotometer at 498 nm is used to quantify the amine group density per cm^2. Briefly, two sets of amino coated substrates were prepared. One set of the substrates was pre-treated by ultrasonication in a 1% solution by weight of SDS, while the other set was not treated and used as it was. All substrates were then immersed in a freshly prepared solution of sSDTB (0.1 mM at pH = 8.0) for 30 minutes at r.t. After incubation, all substrates were thoroughly rinsed with water and then treated with a 37.5 % perchloric acid to allow the formation of 4,4'-dimethoxytrityl cation from the substrates. Since the reaction between the amines and the sSDTB proceeds with 1:1 stiochiometric ratio, the concentration of the released cation was then measured by UV/vis spectrophotometer at 498 nm.

2.4.6 Dual Polarization Interferometry (Farfield)

Dual polarization interferometry (DPI) measures the phase retardation of guided light caused by interaction of the evanescent field with coatings on a top waveguide, by measurement of an interference pattern developed between a sample beam and the unretarded reference beam passing through the bottom waveguide. The different evanescent field penetration depth of light polarised perpendicular- and parallel-to the waveguide surface (TE and TM) are then used to derive refractive index and thickness information about the surface coatings. With this measurement, it is possible to calculate the coating's mass and density. This measurement has the drawback of requiring special substrates but offers some significant advantages for in-situ probing of the interactions of the plasma-formed coatings with aqueous media. These measurements can investigate the structural changes caused to the film by washing with surfactant solutions. The structural changes taking place with the addition of a surfactant were

investigated by monitoring the refractive index, thickness and mass variations following exposure to PBS Tween. DPI measurements have been verified using standard protein systems and have successfully monitored biochemical interactions (e.g. protein adsorption [19; 20] and lipid membrane formation [21; 22].

DPI measurement was carried out using a Farfield AnaLight instrument. This measurement required special substrates, not Zeonor®, but offered some significant advantages for probing in situ the interactions of the plasma-formed coatings with aqueous media. For this measurement, the coatings had to be deposited onto chips whose surface was air-formed silica. The Silicon oxynitride Ana-Chip consists of two optical waveguides, that confine light in defined boundaries, stacked one on top of the other. The substrate is a silicon wafer and the waveguides are silicon dioxide doped with silicon nitride.

2.4.7 Microcontact printing (μCP)

Patterned poly(dimethylsiloxane) (PDMS) stamps were fabricated by pouring a 10:1 (v/v) mixture of Sylgard 184 elastomer and curing agent over a patterned silicon master (stripes of 15x15 μm). The mixture was cured for one hour in the oven at 60º C, then carefully peeled away from the master and left in the oven for another 18 h at 60º C to ensure complete curing.

The covalent attachment of the fluorophore to the amino functionalized Zeonor® substrate was achieved by putting the amino functionalized substrate in contact with a PDMS stamp. Each sample was printed with a new stamp. All the stamps were oxidized for 9 minutes with UV-ozone at room temperature. Immediately after the ozone treatment the stamps were dipped in the ink, a 0.23 M acetonitrile solution of the fluorophore, lissamine rhodamine B sulfonyl chloride, and kept inside for at least 15 minutes. The stamp was removed from the ink, dried with air to remove excess fluorophore solution, and put in contact with the substrate for 3 min before careful removal. The substrates were then extensively rinsed once with a stream of EtOH and once with a stream of demi-water, and dried in an nitrogen stream.

2.4.8 X-Ray Photoelectron Spectroscopy (XPS)

X-Ray photoelectron spectroscopy (XPS) provides a quantitative elemental analysis of plasma deposited coatings. X-rays are directed towards the film causing electrons to be

emitted from various elements in the surface (typically up to approx. 10 nm depth) of the film. By analyzing the energy of the emitted electrons, the chemical composition of the film can be determined. To study the various bonding environments, a high-resolution scan for the core level photoemission spectra of C 1s peak was carried out. The elements present in the coatings can be identified from this XPS survey spectra.

The XPS data were collected on a Kratos Axis UltraDLD equipped with a hemispherical electron energy analyzer. Spectra were excited using monochromatic Al Kα X-rays (1486.69 eV) with the X-ray source operating at 100 W. The measurements were carried out in a normal emission geometry. A charge neutralization system was used to alleviate sample charge buildup, resulting in a shift of approximately 3 eV to lower binding energy. Survey scans were collected with 160 eV pass energy, whilst core level scans were collected with pass energy of 20 eV. The analysis chamber was at pressures in the 10^{-9} Torr range throughout the data collection.

Data analysis was performed using CasaXPS (www.casaXPS.com). Shirley backgrounds were used in the peak fitting. Quantification of survey scans utilized relative sensitivity factors supplied with the instrument. Core level data were fitted using Gaussian-Lorentzian peaks (30 % Lorentzian).

2.4.9 Total Internal Reflection Fluorescence (TIRF) microscopy

Total internal reflection fluorescence (TIRF) microscopy [23; 24] utilizes the evanescent field generated upon TIR to probe fluorescent interactions with a high degree of surface selectivity (typically within approx. 100nm). This technique has been used to investigate the interactions between functionalized fluorescent nanoparticles and the PECVD deposited film in real-time.

3. Results and discussion

3.1 Amine-containing coatings for immobilization of biorecognition elements

Formation of amine containing self-assembled monolayers and polymer-like films is very popular and widely used to modify COP substrates. The relatively high pKa values of amines mean that at physiologically relevant pH, most of the groups are present in their

charge state as –NH3+, which could be advantageous as the biomolecules are also highly charged species. One of the most common precursors used to prepare amine-containing film is aminopropyltriethoxysilane (APTES). A number of reports show that immersing glass or plastic substrates in APTES solution in ethanol leads to formation of an aminated surface. However, such solution-based techniques require the glass or plastic surface to be activated, typically by an oxidation process before reaction with APTES. These multi-step procedures are time consuming, complicated, inappropriate for large-scale production and difficult to reproducibly control the film quality. Gas phase deposition overcomes these drawbacks. However, most published investigations have focused on vapor phase reaction of aminosilanes with an oxidized surface, usually glass or silicon at extremely high temperatures. Such conditions are not suitable for substrates made of plastic material, like COP. PECVD on the other hand, represents a method that combines the substrate activation step with plasma enhanced chemical vapor deposition (PECVD) of an amine-containing precursor.

Chemically, introduction of amines on the surface means that proteins or oligonucleotides can be captured by either physical adsorption or by covalent reaction between the surface and reactive groups on the protein. The latter is usually mediated by using a homo- or hetero-functional cross-linker as depicted on **Fig.1** .

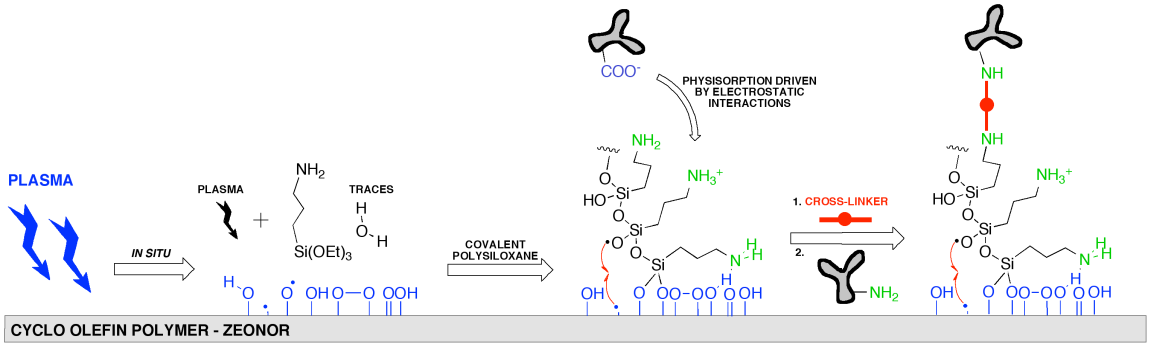

Figure 1 Schematic illustration of the PECVD procedure to obtain amine-containing surface.

The most important characteristics that such prepared coatings should meet is that they should have a high degree of uniformity, high density of functional groups and high stability towards hydrolysis. In addition, the film should be rather hydrophilic with soft-like

property and with high flexibility allowing for more efficient reaction between the antibodies and the surface as well as the analyte. All aforementioned features could be controlled and fine-tuned by range of parameters that are related to the plasma process. In the next section, the consequences of modifying some of the parameters on the coating properties will be discussed.

3.1.1 Influence of plasma process parameters on Functionalization

3.1.1.1 Influence of RF power on Plasma electron density

The hair pin probe is a diagnostic technique for measuring absolute electron density in the discharge using a microwave resonance probe [25]. The principle of the probe is based on measuring the dielectric constant of the plasma surrounding the resonant structure. The probe, consisting of two parallel wires, short circuited at one end and open at the other, resembles a hairpin. The typical length of the hairpin was 1-2 cm. The microwave resonance of the hairpin was used to determine electron density.

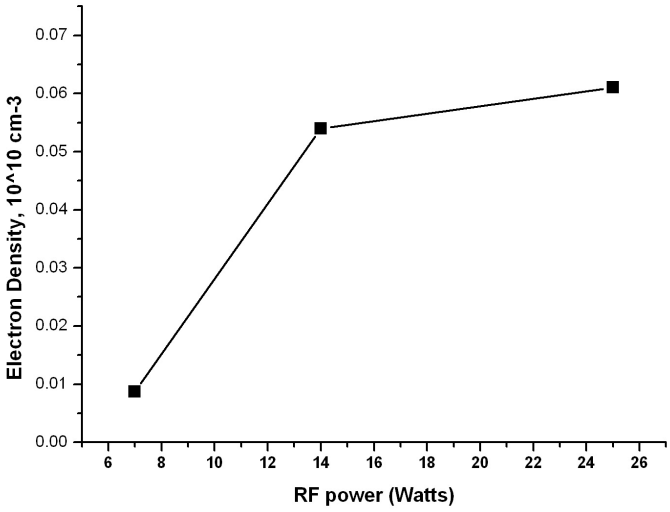

Figure 2 Variation in plasma electron density with input RF power, measured using hair pin probe.

An increase in electron density with RF power (**Fig. 2**) implies that the plasma density increases and hence the fragmentation of the precursor is significantly higher at higher RF power. The fragmentation of the precursor could either be favourable or not, purely on the requirement of the surface characteristics. In the case of inorganic silica-like deposition, a very high electron density is preferred for two reasons (i) a high fragmentation of precursors is required to remove the hydrocarbons from the siloxane/silane precursors so that the resultant film will be silica rich and (ii) with an increase in RF power, the ion bombardment on the growing film will increase resulting in densification of the film [26]. It was also reported that for the deposition of Silanol functional group, the energetic ion bombardment should be minimal and hence the input RF power should be kept to a minimum. It has also been reported that a low RF power is required for plasma deposition of amine functionality. Hence the low power regimes are to be given due consideration.

Also the ion bombardment and hence the surface functionalization depends on whether the films are grown on floating or live electrode as the ion bombardment energy and the ion flux at the substrate is proportional to the input RF power. At the floating substrate, the sheath potential is approximately a few volts below the plasma potential, hence the ion bombardment energy is considerably lower. Hence, in all cases the substrates were placed on the floating electrode.

3.1.1.2 Influence of RF power on amine Functionalization

The influence of the plasma RF power on surface properties, as well as their effect on the reactivity and content of amino groups was investigated. The film thickness measured using ellipsometry showed a linear increase of film thickness with an increase in RF power. An increase in thickness with RF power also implies that the plasma polymerization is more effective with increase in plasma energy. The plasma electron and ion density measured using the hairpin probe (**Fig 2**) showed an increased electron density with an increase in RF power. In order to study the effect of RF power on amine functionalization, a qualitative analysis of the amine content in the surface was carried out by covalently attaching the fluorophore lissamine rhodamine B sulphonyl chloride to

the amine functionality and measuring the fluorescence intensity for the samples deposited at varying RF power (**Fig. 3**).

The amine content increased in the coating increased from 7 watts to 14 watts and reduced drastically at 25 watts. The results indicate that, at the lowest power, the silane was activated and could react with the surface, but was not significantly dissociated. With a higher power of 25 watts, the silane became fragmented, with amine functions being lost and the layer being built through reaction of ethoxy radicals. The key process determinants were to have a sufficient power in the plasma to activate and partially fragment the monomer but not too much as to lose the reactive amine functionality, and sufficient deposition time to develop a reactive layer but not to consume or erode the amine reactivity.

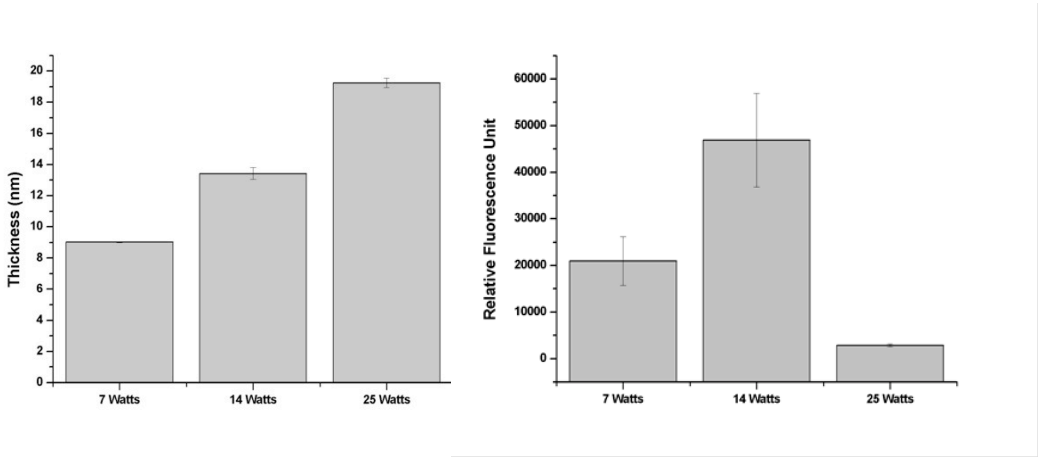

Figure 3 Variation in (left) film thickness and (right) fluorescence intensity of amine functional coatings with input RF power.

3.1.2 Characterization of amine functionalization

To date, a range of methods and analytical tools is available for quantitative and qualitative analysis of $–NH_2$ containing surfaces. Besides the relatively available and routine tools for surface chemists such as ellipsometry, contact angle measurements, FTIR and microscopy, some more specific and somewhat unusual techniques will be discussed in the next section.

3.1.2.1 SIMS

Secondary Ion Mass Spectrometry is based on a pulsed primary ion beam that desorbs and ionizes species from a sample surface. The resulting secondary ions are then accelerated into a mass spectrometer where they are analysed based on their mass/charge ratio.

The secondary ion chemical imaging of the coated samples were taken for $-NH_2^+$ species with m/z = 16 in positive SIMS mode. The measured signal intensity was significantly high suggesting higher quantity of surface amino groups. Also a depth profile measurement was carried out for the same species and the result demonstrated that the amine functionalities were observed both in the surface as well as on the bulk of the coating (**Fig. 4**)

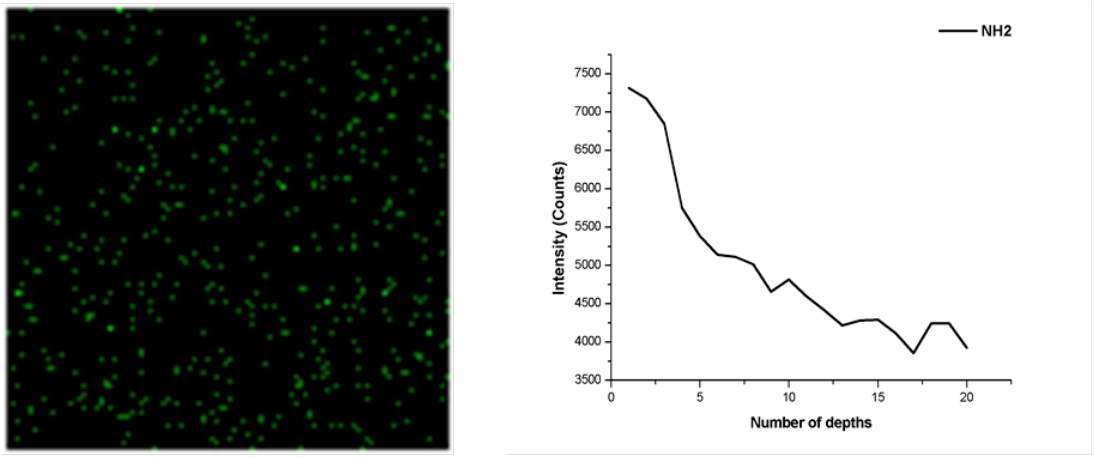

Figure 4 Secondary ion chemical imaging of: (Left) $-NH_2^+$ fragment at m/z = 16 with positive SIMS. (Right) Depth profile analysis of $-NH_2^+$ fragments with positive SIMS. An electron gun charge neutralizer was used to overcome the charging effect**.**

3.1.2.2 Fluorescence intensity of luminophores by microcontact printing; assessment of surface uniformity.

A second fluorescence experiment was performed to unambiguously confirm our previous results and also to test the film homogeneity. Therefore, lissamine rhodamine B

sulphonyl chloride was attached by microcontact printing directly on amino coated Zeonor® surfaces (**Fig. 5**).

The fluorophore attachment to amino-functionalized Zeonor® substrates was achieved by immersing the samples for 1 hour in a 0.23 mM lissamine rhodamine B sulfonyl chloride in acetonitrile. 100 µl triethylamine were added to 100 ml of the lissamine solution. Samples were then rinsed with distilled water and sonicated for 5 minutes in a 0.1 % sodium dodecyl sulfate (SDS) solution. Following this, the substrates were rinsed again with distilled water and dried under a nitrogen stream. The microcontact printing of lissamine fluorophore demonstrates that the coating is homogeneous.

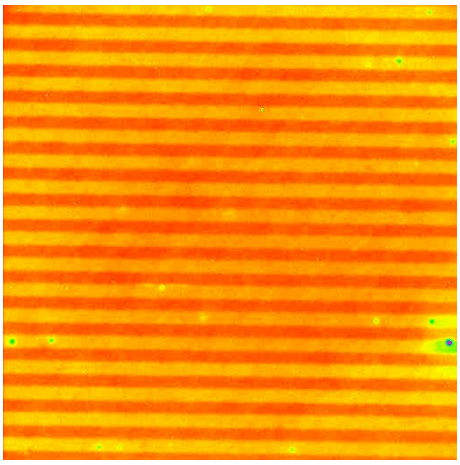

Figure 5. Fluorescence image of lissamine fluorophore attached to amine functionalised coatings by micro contact printing.

3.1.2.3 Quantitative analysis of –NH_2 density by colorimetry and dual polarization interferometry

A simple colorimetric experiment using sSDTB was performed to reveal the number of reactive amino groups on both coatings before and after treatment with PBS Tween. As determined by DPI, the total number of –NH_2 groups per cm^2 present in the coating are $4.82 \pm 0.81 \times 10^{14}$ amine groups/cm^2 and are consistent with the data published on similar systems. It is noteworthy to mention that the value for the maximum amount of amino groups on APTES coated substrates is nearly 78×10^{14} per cm^2 (based on mass of APTES measured by Dual Polarization Interferometry). The experiment with sSDTB

revealed that its succinimidyl ester binds only to about 5% the amines (3.99 x 10^{14}) exposed on the contact (upper) part of the substrate. The remaining 95% of APTES molecules comprise the majority of the bulk polymeric coating, and are presumably involved in a number of non-covalent interactions such as van der Waals forces and hydrogen bonds. Overall, the colorimetric measurement using sSDTB showed that PECVD deposition of APTES yields in a stable and homogeneous coating with high amino group density.

Information about the mass and the density of the adsorbed amine functionalized films were derived, based on which, the amount of amino groups for each sample was calculated, assuming that the silane does not fragment but retains its molecular identity. Therefore, from the mass of the film per cm^2 and the molar mass of the monomer, the number of moles of $–NH_2$ in the film per cm^2 of geometric area can be calculated. DPI allowed monitoring of the layer composition of each slide before and after a treatment with 1% w/v solution of PBS Tween (PBST) to probe the film adhesion and the structural changes upon washing with a detergent. When treated with PBS buffer the mass of amine functionalized film drops from 17.7 ng/cm^2 to 15.1 ng/cm^2 and also the number of amine groups decreased to some extent from 77.77 x 10^{14} amines/cm^2 to 66.43 x 10^{14} amines/cm^2 demonstrating that the loosely bound species are washed away while retaining the reactive functionality on the surface.

3.1.3 Increasing the surface density of amines

It is observed that the siloxane-silicone network could be formed by fragmentation of APTES into aminosiloxane radicals that condense and polymerize on the surface [8; 27]. If these radicals are further fragmented, or the siloxane functionality is not present, then a stable, adherent, amine-reactive network is not formed on COP. The siloxane functionality seems essential both for insertion into C-C bonds of the COP substrate and for building a stable, polymerized network. In order to increase the amine content, ethylene diamine was introduced together with the APTES in the plasma. As a result the amine functionalities from EDA were incorporated into the amino siloxane network deposited from APTES resulting in an increase in the surface amine density. The fluorescence intensity measured by covalent attachment of lissamine fluorophore to the

amine functionality showed that the film deposited by codeposition of APTES+EDA resulted in higher amine content (**Fig. 6**). It is also confirmed by the XPS analysis [8]. The high resolution C1s spectrum of both the coatings showed that the % area of the peak at 286.4 eV in the carbon environment that corresponds to C-N bonding was 36% in the case of films deposited with APTES and 41.7% for the films deposited with APTES+EDA.

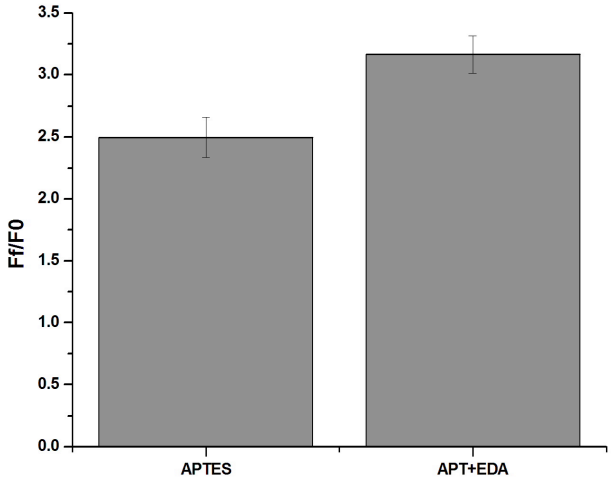

Figure 6 Fluorescence intensity of amine functional coatings deposited by APTES and APTES+EDA

Alteration of the precursor composition, in this case the use of mixtures of APTES and EDA, altered favorably the surface adhesion, the concentration and availability of reactive amine groups. The film wettability of both the films measured by using water contact angle analysis showed that the contact angle was close to 58 degrees for both films.

3.1.4 Reduction of non-specific binding by incorporation of short PEG-like structures

The key challenge in the immuno-sensing field is that sensitivity depends drastically

on the amount of antibodies and their accessibility. Non-specific binding of biomolecules can reduce the sensitivity to a significant extent thereby lowering the performance of the diagnostics devices.

It has been reported that polyethylene glycol (PEG) coating has anti-fouling characteristics that can minimize the non-specific binding [7]. Thompson et. al., have reported that by having alternate long and short chain PEGs the non specific binding has been reduced [28]. Polyethylene glycol, in different forms has proven to be a suitable material for construction of protein-resistant surfaces [29-32]. The low non-specific binding function of PEG-molecules is associated with vast hydration and effects of change in configurational entropy when large molecules adsorb upon them. A numbers of reports describe the preparation of films with crosslinked and non-crosslinked PEG-based matrixes by wet chemistry or vapor methods [33-41].

Therefore, surface coatings that can be mass-produced and combine the quality of low non-specific binding (NSB) and simultaneously have the ability to covalently immobilize biomaterial are of particular interest in biosensor applications. The possibility of increasing the amine contents in the amino silane coating, in which the siloxane acts as an adhesion and network building layer for further functionalization through plasma polymerization, by introducing an additional precursor EDA in the plasma opens up the avenues for incorporating further functionalities into the coating. Diethylene glycol dimethyl ether was co-deposited with APTES so that the PEG-like functionalization was incorporated in APTES plasma polymerized layer. The siloxane layer in APTES acted as an adhesion and network building layer, amine functionality acted as a reactive agent for covalent immobilization of biomolecules and PEG functionality acted as an anti-fouling agent reducing the non specific binding in the bioassay.

In order to test the antifouling characteristics of the plasma deposited layers, green fluorescent protein (GFP) was attached to plasma polymerized APTES, APTES+PEG and PEG-like coatings (**Fig. 7**). The GFP was physisorbed using micro contact printing and the fluorescence intensity was measured after extensive washing with PBS Tween. It was observed that the physisorption was highest on APTES and lowest on PEG. Incorporation of PEG in the APTES layer reduced the non-specific binding to a significant extent.

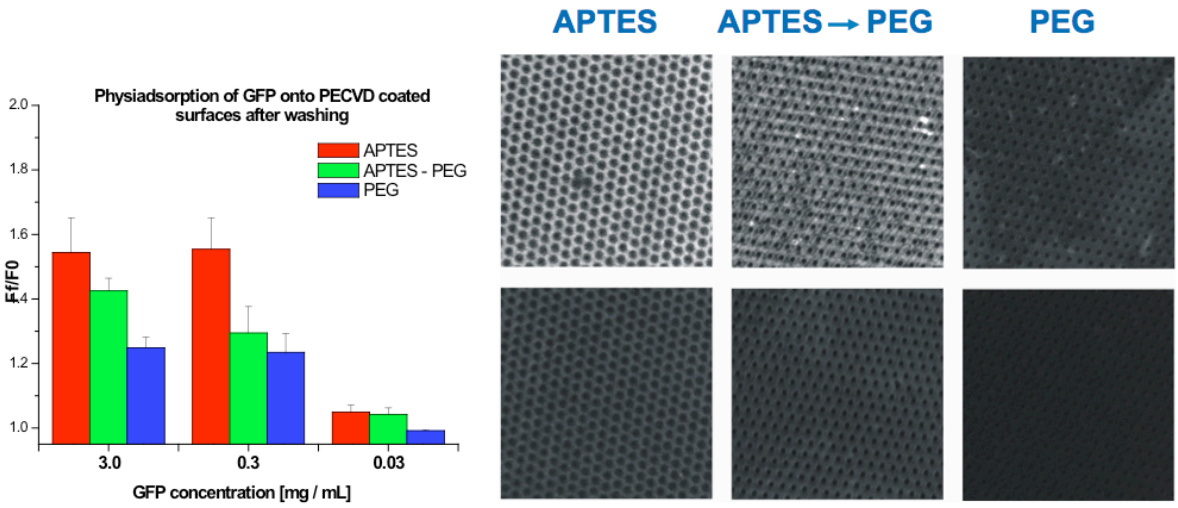

Figure 7 Fluorescence intensities of Green Fluorescent Protein transferred from PDMS stamps onto COP substrates treated with APTES, APTES-PEG and PEG chemistries. Top images – GFP printed at 0.03 mg/mL, before washing, Bottom images – GFP printed at 0.03 mg/mL after washing

For the immobilization of antibody, 1 µl volume (0.2 mg/ml) of cy5 labeled Anti Human IgG (AHIgG) was printed on a PEG coated and APTES + PEG coated Zeonor® slide and incubated at 37 ◦C for 1 h. The surfaces were then washed with PBS–Tween 20 (0.2%, v/v) and subsequently with PBS. The detection was carried out by measuring the fluorescence intensity (FI) with fluorescence microscopy. The fluorescence intensities are reported as the means of the intensities from three different spots. The fluorescence intensity of PEG-like coating was significantly lower than that of APTES+PEG due to the anti-fouling characteristics (**Fig.8**).

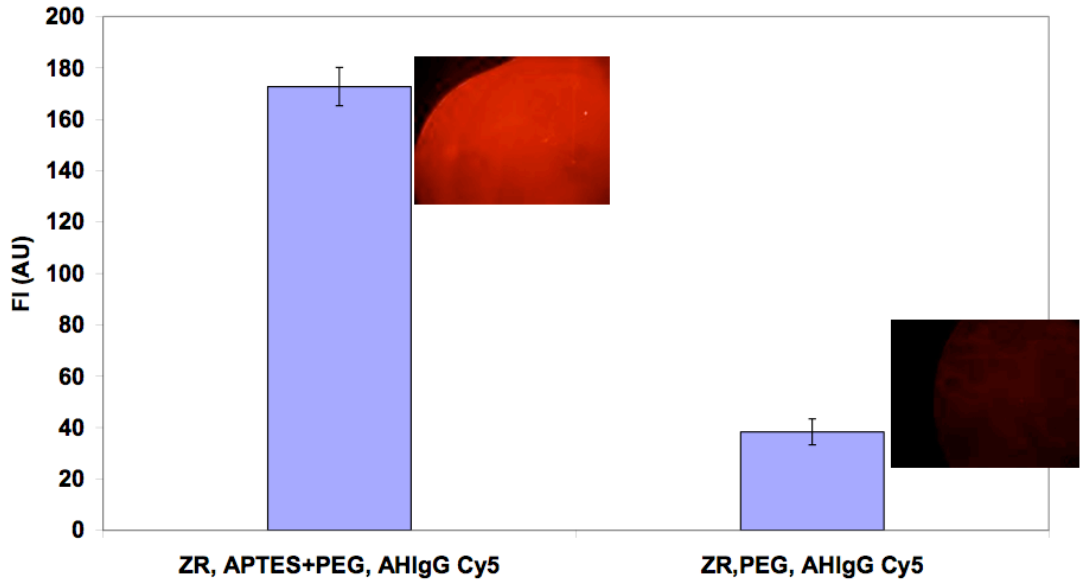

Figure 8 Fluorescence intensity measurement of APTES+PEG and PEG coated Zeonor® samples attached with cy5 labeled Anti Human IgG

3.2 Thiol-containing coatings for immobilization of biorecognition elements

Because of their conductivity, reflectivity, and robustness, gold and silver are often used as transducing surfaces in biosensors. The binding of Au or Ag to organosulfur molecules like thiols or sulfides represents a chemisorption system with unique and interesting properties. The initial steps of the self assembly process and the structural properties of the resulting self assembled monolayers (SAMs) are still a matter of intense current research effort. Formation of SAMs has been widely studied by surface characterization techniques [42] and their building on gold surfaces allows control of the amount and orientation of the antibodies.

There is a need for an alternative method that can improve the functionalization of the surface with the thiol group. This would also be suitable for bulk processing and would be a relatively cost effective process. PECVD has been explored for the deposition of the thiol functional groups. Plasma deposited thiol functionality can potentially be used for binding gold or silver nanoparticles which in turn can be used for signal amplication in bioassays (**Fig.9**).

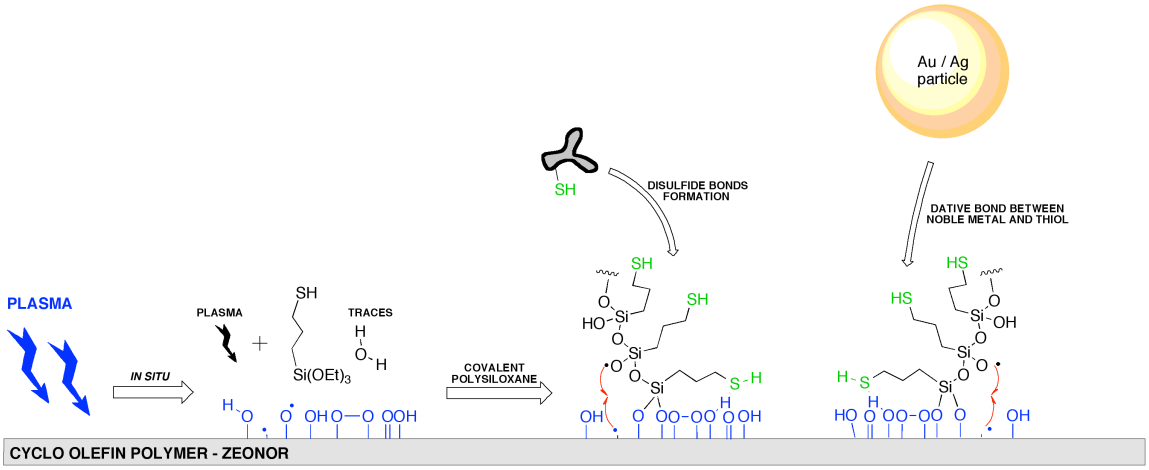

Figure 9 Schematic illustration of the PECVD procedure to obtain thiol-containing surface. Cysteine residues in proteins can be used to form disulfide bond between the molecule and the thiol-containing surface. Also, noble metals, such as silver and gold

readily react with –SH groups to form very stable dative bond. The energy of the metal-S bond is comparable to that of covalent bond.

3.2.1.1 XPS of mercapto silane on zeonor

X-ray photoelectron spectroscopy (XPS) was carried out for a quantitative elemental analysis of the plasma deposited coatings. The elements present in the coatings were identified and quantified from XPS survey spectra (**Fig 10**). For further analysis, high-resolution spectra were recorded from individual peaks. Quantitative analysis confirmed the presence of 11 Atomic percentage of S in the coating.

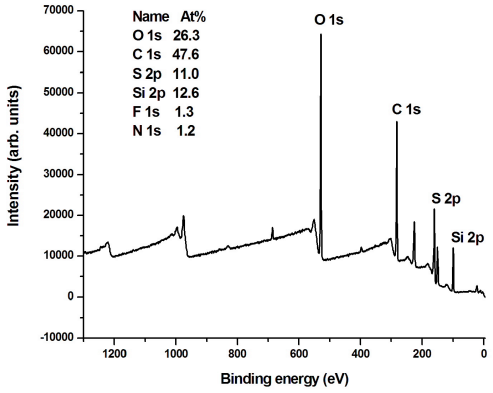

Figure 10. XPS survey scan of mercapto silane coatings taken with a pass energy of 160eV

3.2.1.2 Covalent attachment of Iodoacetamide dye and Silver Nanoparticles to mercapto functionality

The fluorophore attachment to mercapto-functionalized Zeonor substrates was achieved by spotting the samples with NIR664- Iodoacetamide for 2 hours. Samples were then sonicated for 1 minute in PBS solution, followed by a rinse with distilled water and dried under a stream of nitrogen before every scan. The presence of high fluorescence intensity after several sonication steps demonstrates that the mercapto silane has a good adhesion to the COP substrate and also the reactivity of mercapto functionality is

still retained after plasma deposition. The plasma polymerized mercapto silane coating containing highly reactive free SH group can be used for both binding gold/silver nanoparticles for signal amplication in bioassays as demonstrated in **Fig. 11** and also be used for specific binding of biomolecules through disulphide linkage.

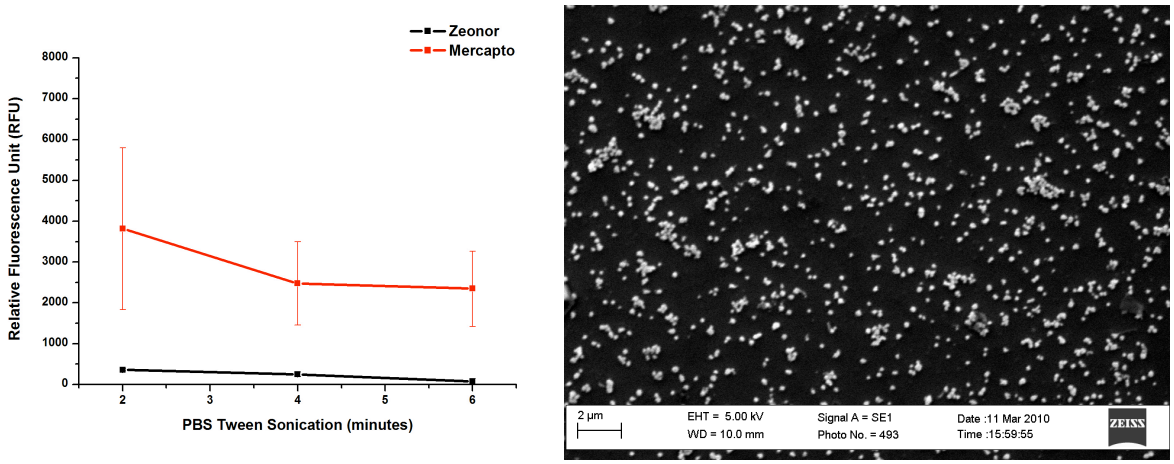

Figure 11 (Left) fluorescence intensity of iodo acetamide fluorophore attached to mercapto functionality and (Right) SEM image of silver nanoparticle attached to mercapto functionality.

4. Conclusion

Surface functionalization of COP through plasma polymerization has been effectively demonstrated by depositing reactive amine coatings, repellant PEG-like coatings and mercapto functionality. By codepositing APTES and EDA, the surface amine concentration was increased and by co-depositing APTES+PEG the non-specific binding was reduced by incorporating the PEG functionality in the aminosilane layer. The presence of reactive amine functionality along with repellant PEG coating enabled covalent immobilization of biomolecules with reduced non-specific binding. We identified the effect of plasma power in both activating and fragmenting the aminosilane. The consequences were that, in our system, whilst the thickness of the layer could be increased by increasing power, the chemical functionality was optimal at a specific

combination of parameters. We speculated that the effect of fragmentation of the silane into siloxane, ethoxy and alkyl amine radicals was the determining factor.

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